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Receptors Suggest Multiple Mechanisms in Patterning of the Visual System

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The EphA3 receptor tyrosine kinase has been implicated in guiding the axons of retinal ganglion cells as they extend in the optic tectum. A repulsive mechanism involving opposing gradients of the EphA3 receptor on retinal axons and its ligands, ephrin-A2 and ephrin-A5, in the tectum influences topographic mapping of the retinotectal projection. To investigate the overall role of the Eph family in patterning of the visual system, we have used *in situ* hybridization to localize nine Eph receptors in the chicken retina and optic tectum at Embryonic Day 8. Three of the receptors examined correspond to the novel chicken homologs of EphA2, EphA6, and EphA7. Unexpectedly, we found that many Eph receptors are expressed not only in retinal ganglion cells, but also in tectal cells. In particular, EphA3 mRNA is prominently expressed in the anterior tectum, with a pattern reciprocal to that of ephrin-A2 and ephrin-A5. Similarly, ephrin-A5 is expressed not only in tectal cells but also in the nasal retina, with a pattern reciprocal to that of its receptor EphA3 and partially overlapping with that of its other receptor EphA4. Consistent with the even distribution of EphA4 and the polarized distribution of EphA4 ligands in the retina, probing EphA4 immunoprecipitates from different sectors of the retina with anti-phosphotyrosine antibodies revealed spatial differences in receptor phosphorylation. These complex patterns of expression and tyrosine phosphorylation suggest that Eph receptors and ephrins contribute to establishing topography of retinal axons through multiple mechanisms, in addition to playing a role in intraretinal and intratectal organization.

INTRODUCTION

The mechanisms responsible for the complex organization of neuronal connections in the developing visual system have been the subject of investigation for many years. The advancing growth cones of retinal ganglion cell axons encounter environmental cues that guide them from the eye to their targets in the optic tectum in a spatially organized manner. These cues may occur in two forms: first, as long-range cues in the form of secreted diffusible factors, and second, as short-range cues in the form of contact-mediated signals providing more precise positional information (Tessier-Lavigne and Goodman, 1996). Sperry postulated that positional information in the retinotectal system could be obtained by having at least two superimposed orthogonal gradients of cytochemical molecules in the retina and tectum, which would provide the coordinates defining cell position (Sperry, 1963). His chemoaffinity theory predicted that retinal ganglion cell axons find their synaptic targets in the tectum through a process of homophilic interaction between recognition molecules present on their growth cones and on tectal neurons.

A number of proteins have been identified whose expression in the retina and tectum is not uniform, suggesting that they may convey the type of topographical information predicted by Sperry (reviewed by Kaprielian and Patterson, 1994). In particular, EphA3 (Cek4), a member of the Eph family of receptor tyrosine kinases, is expressed in an increasing nasal to temporal (anterior to posterior) gradient in chicken retinal ganglion cells (Cheng *et al.*, 1995). EphA3 is one of 14 related Eph receptors (van der Geer *et al.*, 1994), which have the distinctive property of binding membrane-anchored ligands (reviewed by Pandey *et al.*, 1995). Five of the ligands (belonging to the ephrin-A subclass) are linked to the plasma membrane through a glycosylphosphatidylinositol (GPI) linkage, while three (belonging to the ephrin-B subclass) are transmembrane proteins (Eph Nomenclature Committee, 1997). Although the ligands are promiscuous in their binding to Eph receptors, some specificity has been demonstrated (Gale *et al.*, 1996). Ligands of the ephrin-A subclass bind preferentially to a subset of the Eph receptors, now known as the EphA receptors (Eph Nomenclature Committee, 1997) and ligands of the ephrin-B subclass bind preferentially to a different subset of Eph receptors, now known as the EphB receptors.

Although not unique in its expression gradient (Kaprielian and Patterson, 1994), the receptor EphA3 has generated interest as a candidate molecule involved in neuronal path-

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finding based on its graded distribution in the retina, and the graded distribution of its ligands, ephrin-A2 (ELF1) and ephrin-A5 (RAGS), in the optic tectum (Drescher *et al.*, 1995; Cheng *et al.*, 1995). Avoiding the high concentrations of the ligands ephrin-A2 and ephrin-A5 present in the posterior tectum, the axons of temporal retinal ganglion cells, which express high levels of EphA3, terminate in the anterior portion of the optic tectum. The opposing distributions of EphA3 on retinal ganglion cell axons and its ligands in the tectum suggested a guidance mechanism for these axons similar to that proposed by Sperry, but based on contact-mediated repulsion rather than homophilic interactions. *In vitro* and *in vivo* experiments have confirmed that both ephrin-A2 and ephrin-A5 have the properties of repulsive guidance proteins for retinal ganglion cell axons (Drescher *et al.*, 1995; Nakamoto *et al.*, 1996).

Another Eph receptor, EphB2 (Cek5 and Qek5), is more highly concentrated in the ventral retina than in the dorsal retina (Holash and Pasquale, 1995; Kenny *et al.*, 1995). It was, therefore, hypothesized that this receptor may guide retinal ganglion cell axons to avoid high concentrations of a ligand that would be concentrated in the ventral tectum (Brambilla and Klein, 1995; Friedman and O'Leary, 1996). Unexpectedly, however, ephrin-B1—a ligand for EphB2—was found to be expressed at low levels and evenly in the optic tectum rather than concentrated in the ventral portion (Holash *et al.*, 1997). Interestingly, the ligands ephrin-B1 and ephrin-B2 were both detected in the retina, where they are concentrated in the dorsal aspect (Marcus *et al.*, 1996; Holash *et al.*, 1997). Their polarized and reciprocal patterns of expression in the retina suggest that EphB2 and its ligands influence the organization of the visual system along the dorsal–ventral axis, but presumably according to a mechanism that is different from that underlying guidance mediated by EphA3 and its ligands.

A recent mutagenesis screen in zebrafish indicated that there are many distinct genes that regulate axon topography in the visual system (Baier *et al.*, 1996; Trowe *et al.*, 1996). Mutations of several genes affected mapping of zebrafish retinal axons on the surface of the optic tectum. These genes, therefore, presumably represent proteins that provide positional information in the tectum, consistent with Sperry's chemoaffinity theory. Mutations of other genes, however, influenced the directional growth of retinal axons in the retina or their sorting in the optic tract (Baier *et al.*, 1996; Karlstrom *et al.*, 1996), suggesting that topography in the visual system is regulated by a combination of genes that act in different parts of the optic pathway. Although the identity of the genes that were mutagenized is unknown, Eph receptors and ephrins are likely candidates as molecules that regulate the topographic organization of the visual system.

To determine whether other Eph receptors, in addition to EphA3 and EphB2, have distributions consistent with their influencing aspects of retinotectal topography, we have compared the expression in the retina and optic tectum of three receptors of the EphB group and six receptors of the EphA group, including the three previously unknown chicken homologs of EphA2, EphA6, and EphA7. We found that many Eph receptors are expressed at substantial levels in the chicken visual system at Embryonic Day 8, when retinal ganglion cell axons are growing toward their tectal targets. However, the Eph receptors examined are not exclusively expressed in retinal cells, as would be expected if their role in the visual system was limited to targeting the growth cones of retinal ganglion cells to tectal regions specified by a particular ligand concentration. Similarly, the ephrin ligands are not exclusively expressed in tectal cells, as would be expected if their role was limited to specifying positional identity of tectal target cells. We detected complementary as well as overlapping patterns of Eph receptors and ephrin mRNA expression in both the retina and the tectum. Our results suggest that Eph receptors and ephrins regulate the organization of the visual system in a complex manner, in agreement with certain theoretical models (Fraser and Perkel, 1990) that predicted the existence of multiple mechanisms operating in concert to accurately guide the axons of the retinotectal projection.

MATERIALS AND METHODS

Amplification of Chicken Eph Receptor Sequences

Two degenerate primers were designed based on highly conserved stretches of amino acids in the extracellular domain of the Eph receptors. The forward primer, TG(T/C)AA(G/A)GA(G/A)AC(G/A/T/C)TT(T/C)A, encodes the amino acid sequence CKETFN (corresponding to amino acids 115–120 of chicken EphB2 (Pasquale, 1991)); the reverse primer, TC(T/C)TG(G/A)AA(G/A/T/C)GC(G/A/T/C)A(G/A)(G/A), encodes the amino acid sequence FYLAFQ (corresponding to amino acids 182–187 of chicken EphB2). PCR amplification was performed with the degenerate primers by using 35 cycles of the following program: 1.5 min at 95°C; 1.5 min at 42°C; 2 min at 72°C.

Plasmids containing chicken EphA3, EphA4, EphA5, EphA7 (see below), EphB1, EphB2, and EphB3 cDNAs were used as templates for amplification of fragments with sizes of approximately 210 bp. Sequences corresponding to the previously unknown chicken EphA6 receptor were amplified with the degenerate primers from chicken genomic DNA. Sequences corresponding to the previously unknown chicken EphA7 receptor were amplified using the forward primer GTAGAATTGAAATTCACC (corresponding to residues 483–500 of human EphA7 (GenBank L36642)) and the reverse primer CCAGCACTTCTTGTAGTA (corresponding to residues 786–803 of human EphA7). Amplification of genomic DNA with these primers using the same conditions described above for the degenerate primers yielded a 320-bp product. Sequences corresponding to the previously unknown chicken EphA2 receptor were amplified using the forward primer TCCTGCAAGGAGACCTTCAC (corresponding to residues 405–424 of human EphB4 (Bennett *et al.*, 1994)) and the reverse primer GTCAGGTTCACAGTCAGCTG (corresponding to residues 684–703 of human EphB4). Amplification of an adult chicken brain cDNA library (Clontech) with these primers using the same conditions as described above for the degenerate primers yielded a 298-bp product, which was further amplified with the degenerate primers.

Amplified products of the expected sizes were gel-purified and ligated into the TA vector pCR2.1 (Invitrogen) and plasmids were

screened for the presence of the inserts in either the sense or antisense orientation with respect to the T7 promoter. Since the chicken EphA2, EphA6, and EphA7 PCR products were obtained only in one orientation in the vector pCR2.1, the inserts were removed by digestion with *Xba*I and *Hin*dIII and subcloned into the vector pBluescript (SK^+) (Stratagene) to obtain them in the opposite orientation. Plasmids containing the inserts were sequenced by the dideoxy-termination method (USB).

Preparation of Riboprobes

Sense and antisense RNA probes for the various Eph receptors were prepared using a Riboprobe kit (Promega) and T7 RNA polymerase. The plasmids containing the regions amplified with degenerate primers were used as templates for the transcription reactions. In addition, plasmids containing nucleotides 748–1724 of chicken EphA3 (Sajjadi *et al.*, 1991), 2527–3132 of chicken EphB1 (GenBank Z19110), 2938–3591 of chicken EphB3 (GenBank Z19061), and 152–420 of RAGS (Drescher *et al.*, 1995) were also used to prepare probes. Ten micrograms of template DNA was linearized using an appropriate restriction enzyme, phenol extracted, and ethanol precipitated. The linearized templates were transcribed with T7 RNA polymerase in the presence of 50 μCi [α-^{33}P]UTP (2000 Ci/mmol, New England Nuclear) for 1 h at 37°C, according to the specifications of the manufacturer (Promega) for the preparation of high specific activity probes. The reaction volume was reduced from 20 to 15 μl. The templates were removed by a 30-min digestion with DNase I followed by phenol/chloroform extraction. Unincorporated ribonucleotides were removed by using CHROMA SPIN-10 columns (Clontech), and the riboprobes were quantitated by scintillation counting.

In Situ Hybridization

Embryonic Day 8 chicken embryo heads were fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Eight-micrometer sections were mounted on Fisher Superfrost Plus slides and used for hybridization with ^{33}P-labeled riboprobes. *In situ* hybridization was performed according to standard protocols (Simmons *et al.*, 1989; Holash *et al.*, 1997). Briefly, deparaffinized sections were deproteinated using 0.2 M HCl, digested with proteinase K (2.5 μg/ml), acetylated with 0.1% acetic anhydride, dehydrated, and air-dried prior to hybridization. Sections were hybridized overnight at 50°C in hybridization buffer (300 mM NaCl, 20 mM Tris, pH 8.0, 5 mM EDTA, 1× Denhardt's, 0.2% SDS, 10 mM DTT, 0.25 mg/ml tRNA, 50% formamide, 10% dextran sulfate) containing riboprobe (5×10^4 dpm/μl). Following hybridization, the slides were immersed in 4× SSC (150 mM NaCl, 15 mM sodium citrate·$2H_2O$), washed in 2× SSC (2 × 15 min, 55°C), digested with RNase A (20 μg/ml) for 30 min at 37°C, and incubated for 30 min in RNase buffer at 37°C. The slides were then washed for 30 min at 65°C and 10 min at room temperature in 0.1× SSC. Following dehydration, the slides were air-dried and exposed to X-ray film for 2–4 days, followed by dipping in photographic emulsion for 2–4 weeks. Following development of the emulsion, the slides were counterstained with 0.1% cresyl violet in H_2O. Photography was performed with a Nikon Optishot microscope using dark-field and bright-field imaging.

Immunoprecipitation and Immunoblotting

Retinal and tectal tissues were dissected from Embryonic Day 8 chicken embryos and stored frozen at −80°C. They were then sonicated in RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml pepstatin, and 10 μg/ml leupeptin, 1 mM sodium orthovanadate (Sigma), 10 mM sodium pyrophosphate (Sigma), and 100 mM sodium fluoride (Sigma) and protein concentrations were measured using a protein assay kit (Bio-Rad). For immunoprecipitations, aliquots containing equal amounts of protein (approximately one retina) were precleared with 10 μl Staph A (Boehringer Mannheim) and incubated for 40 min with 10 μg of anti-EphA4 antibodies (Soans *et al.*, 1994) or anti-EphA3 antibodies (Soans *et al.*, 1994; Monschau *et al.*, 1997) absorbed on 10 μl Staph A. The immunoprecipitated material was washed three times with RIPA buffer and once with PBS. SDS-containing sample buffer was added, and the immunoprecipitates were boiled for 5 min.

For immunoblotting, proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). After transfer, the filters were blocked for 1 to 16 h in 3% BSA in TBS (Tris–hydroxyethyl aminoethane-buffered saline) and then incubated for approximately 4 h at room temperature, or overnight at 4°C, with 3 μg/ml anti-phosphotyrosine antibodies (Maher and Pasquale, 1988). After several washes with TBS, the filters were incubated with 0.2 μg/ml protein A peroxidase (Sigma) in TBS containing 3% BSA for 1 h. The filters were then washed and developed using enhanced chemiluminescence reagents (Amersham). For reprobing, the filters were washed, incubated in 3% BSA containing 0.2% sodium azide, and reprobed with anti-EphA4 or anti-EphA3 antibodies at a concentration of 1 μg/ml.

RESULTS

Preparation of in Situ Hybridization Probes Corresponding to Novel as Well as Previously Known Chicken Eph Receptors

To systematically examine the expression patterns of Eph receptors in the developing chicken visual system, a panel of riboprobes for *in situ* hybridization was required. To facilitate the preparation of DNA templates that could be used to generate ^{33}P-labeled riboprobes corresponding to a number of different Eph receptors, we devised a strategy based on PCR amplification with degenerate primers. Alignment of the amino acid sequences of the seven previously known chicken Eph receptors with mammalian EphA2 (Eck), EphA6 (Ehk2), EphA7 (Ehk3), and EphB4 (Htk) revealed two conserved stretches of amino acids in the extracellular regions: CKETFN and FYLAFQ (Fig. 1A). These amino acid sequences were compatible with the preparation of primers of relatively low degeneracy and sufficient length for PCR amplification. The EphA8 (Eek) receptor, whose sequence in this region has only recently become available (Park and Sánchez, 1997), also contains these conserved stretches. In contrast, the sequences of EphA1 (Eph) (Hirai *et al.*, 1987) and EphB6 (Mep) (Gurniak and Berg, 1996) are somewhat divergent, and they may be amplified less efficiently. The degenerate primers were used for PCR amplification of the cDNAs encoding the previously identified chicken Eph receptors (Pasquale, 1991; Sajjadi *et al.*, 1991; Sajjadi and Pasquale, 1993). Since both degenerate primers correspond to sequences contained in exon 3 of chicken EphB2 (Cek5) (Connor and Pasquale, 1995), they were also used to amplify chicken genomic DNA. Among several Eph receptor se-

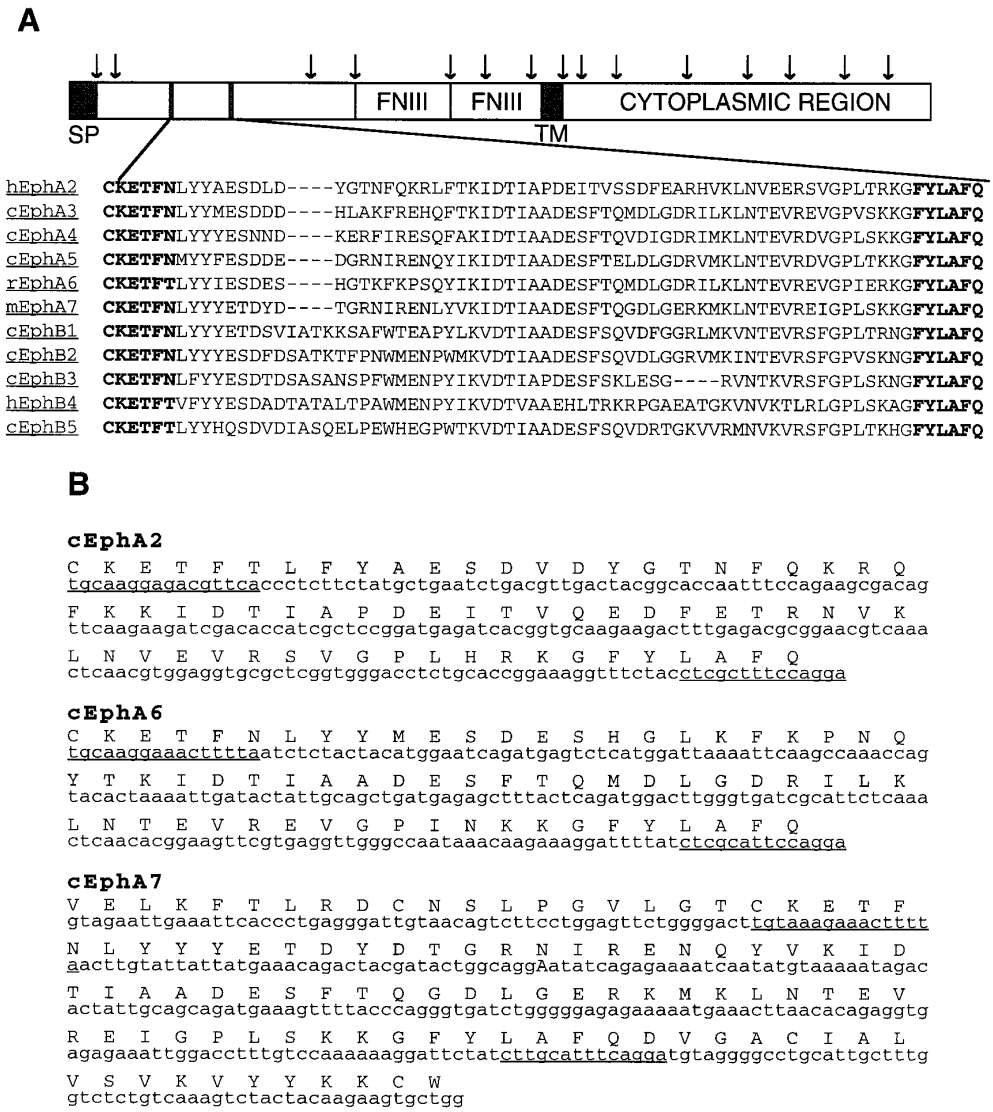


FIG. 1. PCR amplification of Eph receptor sequences with degenerate primers and identification of novel chicken receptors. (A) Two conserved stretches of amino acids suitable for the preparation of degenerate primers (shown in bold) were identified in the extracellular domains of receptors of the Eph family. The receptor sequences used for alignment and their location with respect to the domain structure of an Eph receptor are indicated. The letter preceding the name of a receptor indicates the species of origin: h, human; c, chicken; r, rat; and m, mouse. These sequences correspond to the region used to prepare riboprobes for *in situ* hybridization. The positions of introns in the chicken EphB2 receptor (Connor and Pasquale, 1995) are indicated by arrows. SP, signal peptide; FNIII, fibronectin type III repeat; TM, transmembrane region. (B) Partial sequences of newly identified chicken Eph receptors. The cDNA sequences and corresponding amino acid sequences of cloned PCR products are shown. The sequences of the degenerate primers are underlined.

quences obtained from amplification of genomic DNA, one was novel and most closely related to mammalian EphA6 (Fig. 1B, middle sequence).

Primers having nucleotide sequences conserved in mouse, rat, and human EphA7 (Ciossek *et al.*, 1995; Fox *et al.*, 1995; Valenzuela *et al.*, 1995), and flanking the degenerate primers, were also used for amplification of chicken genomic DNA under low stringency conditions. A novel amplified product most closely related to EphA7 was obtained (Fig. 1B, bottom sequence), which was further amplified with the degenerate primers. Another PCR product was obtained from amplification under low stringency conditions of an adult chicken brain cDNA library with primers flanking the degenerate primers and based on nucleotide sequences conserved in mouse and human EphB4 (Bennett *et al.*, 1994), followed by reamplification with the degenerate primers. Its sequence was most closely related to that of EphA2 rather than to that of EphB4 (Fig. 1B, top sequence).

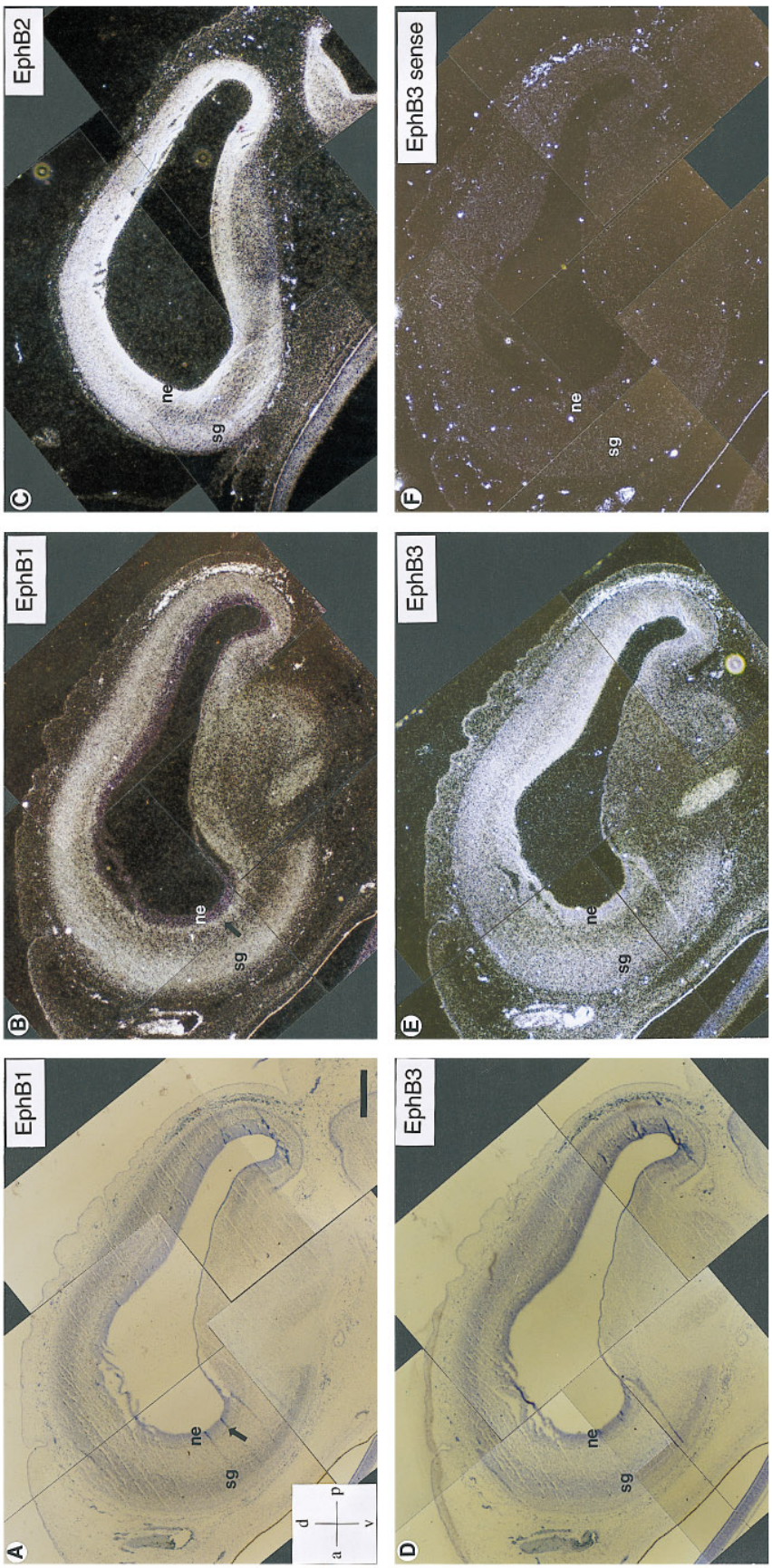


FIG. 2. Expression of EphB receptors in sagittal sections of the chicken optic tectum at Embryonic Day 8. In dark-field photographs of *in situ* hybridization experiments, EphB1 (B) was detected most prominently in layer II or stratum griseum centrale (sg) (LaVail and Cowan, 1971), and in the subventricular zone (indicated by an arrow). The neuroepithelial layer (ne), which is the innermost layer containing the highest concentration of cells, did not appear labeled. Layer I, a cell-sparse layer that separates the subventricular zone and the stratum griseum centrale and contains cells that are migrating outward, was labeled at lower levels. In contrast, EphB2 (C) and EphB3 (E) were primarily detected in the neuroepithelial layer, and only at lower levels in the stratum griseum centrale. All the EphB receptors examined appeared to be evenly expressed with respect to the dorsal–ventral and anterior–posterior axes, except for differences due to differentiation or the absence of layers in the central portion of the ventral tectum in the sections shown in B and E. The EphB1 riboprobe used corresponds to nucleotides 2527–3132 and the EphB3 riboprobe corresponds to nucleotides 2538–3591. However, similar results were obtained with riboprobes corresponding to the regions amplified with the degenerate primers. A and D are bright-field photographs of B and E, respectively, and show the cresyl violet staining. Axes of the tectum are as indicated in A: a, anterior; p, posterior; d, dorsal; v, ventral. Scale bar in A, 500 μm.

The nucleotide sequences of the regions amplified with the degenerate primers were sufficiently divergent (51.6 to 76.5% identity) to allow the preparation of *in situ* hybridization probes specific for each receptor (see below). In some experiments, we also used probes corresponding to other regions of several Eph receptors to further confirm the specificity of the hybridization signals. A phylogenetic tree, constructed using the nucleotide sequences of the regions flanked by the degenerate primers, confirmed that the three novel chicken cDNAs most likely represent chicken EphA2, EphA6, and EphA7 (not shown).

Distribution of EphB Receptors in the Retina and Optic Tectum

To examine the expression of EphB receptors in the developing chicken visual system at Embryonic Day 8, probes for EphB1 (Cek6), EphB2 (Cek5), and EphB3 (Cek10) were used for *in situ* hybridization of sagittal sections of chicken embryo heads containing both the retina and the optic tectum. Interestingly, the signals for EphB1 and EphB3 were substantially more prominent in the tectum than in the retina, whereas that for EphB2 was more prominent in the retina (not shown).

In dark-field photographs of the optic tectum (Fig. 2), EphB1, EphB2, and EphB3 appeared to be evenly expressed with respect to the anterior–posterior and dorsal–ventral axes, except for differences that correlate with the uneven differentiation of the layers (La Vail and Cowan, 1971) or the absence of some layers in portions of the ventral tectum. The expression patterns of the three EphB receptors, however, were different. EphB1 transcripts (Fig. 2B) were highly concentrated in layer II, one of the more external layers of the tectum, which will give rise to the stratum griseum centrale (sg) (LaVail and Cowan, 1971). This layer, which is wider in the more differentiated anterior tectum, originates from the migration of postmitotic neurons from the neuroepithelial layer (ne), which is the innermost layer of the tectum. EphB1 mRNA was also observed in a thin layer of cells just external to the neuroepithelial layer (arrows in Figs. 2A and 2B). These EphB1-positive cells presumably represent postmitotic neuroblasts of the subventricular zone (Puelles *et al.*, 1978), which accumulate just peripherally to the neuroepithelial layer before migrating outward across layer I to the stratum griseum centrale. Both EphB2 and EphB3 were highly expressed in the innermost region of the tectum, corresponding to the neuroepithelial layer (Figs. 2C and 2E), and present at lower levels in the developing stratum griseum centrale.

In the retina, the signal for EphB1 was strongest in a layer (arrows, Figs. 3A and 3C) that was determined to be the retinal ganglion cell layer by comparison with bright-field images (Figs. 3B and 3D). EphB1 mRNA was detected at approximately equal levels in the dorsal and ventral retina (Figs. 3A and 3C) as well as in the nasal and temporal retina (not shown). Unlike EphB1, both EphB2 and EphB3 were detected at lower levels in the dorsal retina (Figs. 3G and 3H) compared to the ventral retina (Figs. 3I and 3J) and were distributed throughout the retinal layers.

Distribution of EphA Receptors in the Retina and Optic Tectum

To examine the expression of EphA receptors in the developing chicken visual system at Embryonic Day 8, probes for EphA2 (Eck), EphA3 (Cek4), EphA4 (Cek8), EphA5 (Cek7), EphA6 (Ehk2), and EphA7 (Ehk3) were used for *in situ* hybridization of sagittal sections of chicken embryo heads. Unexpectedly, EphA3 was found to be expressed in the optic tectum at considerably higher levels than in the retina. Furthermore, in the tectum EphA3 transcripts were concentrated in the anterior portion of the neuroepithelial layer, subventricular zone, and developing stratum griseum centrale (Figs. 4A and 4B). In addition, EphA3 was detected at considerable levels in regions external to the tectum, particularly in the meninges, the connective tissue underneath the tectum, and a discrete area of the telencephalon, in addition to the telencephalic ventricular layer (Fig. 4A).

The other EphA receptors were all found to be expressed in the developing stratum griseum centrale (Figs. 4C, 4E, 4G, and 4I), with the exception of EphA2, which was not detectably expressed in the tectum (not shown). In some sections, two bands of higher expression were observed within the stratum griseum centrale (see, for example, Figs. 4E and 4G). Only EphA5 appeared to be distributed in an increasing posterior to anterior gradient, which was most noticeable in the neuroepithelial layer (Fig. 4C). Due to the low expression of EphA5, EphA6, and EphA7, gradients of expression in the stratum griseum centrale, which is more prominent in the more differentiated anterior tectum (Figs. 4D, 4F, and 4H), were difficult to evaluate. Other differences in the expression patterns of these receptors were apparent. EphA5, EphA6, and EphA7 were all found to be present in the subventricular zone, like EphB1, but only the EphA5 signal extended into the ventral portion of the tectum (Fig. 4C). EphA6 expression in the neuroepithelial layer of the tectum was not detectable (Fig. 4E). In the telencephalon, this receptor exhibited a discrete domain of expression different from that of EphA3 (Fig. 4E). Expression of EphA7 was low and more even across all the layers of the tectum and throughout the telencephalon (Fig. 4G). EphA4 was more prominently expressed in the neuroepithelial layer than the stratum griseum centrale and evenly with respect to the anterior–posterior and dorsal–ventral axis (Fig. 4I).

In the retina, EphA3 was more highly expressed in the temporal than the nasal retina (not shown), as previously reported (Cheng *et al.*, 1995). The EphA5, EphA6, and EphA7 receptors were detected primarily in the retinal ganglion cell layer (insets in Figs. 4C, 4E, and 4G) and the signal observed with the antisense EphA2 probe was similar to that with the corresponding sense probe (not shown). Although hybridization signals for EphA5, EphA6, and EphA7 were higher in the temporal than the nasal retina (not shown), a higher degree of stratification into layers was observed in the temporal retina (see Fig. 5), which is consistent with a higher degree of differentiation (Mey and Thanos, 1992; Rager *et al.*, 1993). It is therefore possible that expression of EphA5, EphA6, and EphA7 in retinal ganglion cells is upregulated during retina differentiation.

Ephrin-A Subclass Ligands in the Retina

Since the EphA receptors examined were found to be expressed not only in retinal cells but also in tectal cells, we examined whether ephrin-A ligands are present in the retina, in addition to the tectum. By using reverse-transcription–PCR with degenerate primers to amplify ephrin-A sequences from E8 chicken retina mRNA (unpublished data), we found that ligands of the ephrin-A subclass are indeed present in the retina, with many of the amplified fragments corresponding to ephrin-A5 (unpublished data). By *in situ* hybridization, ephrin-A5 appeared to be expressed throughout the nasal retina (Fig. 5E) at levels higher than in the temporal retina (Fig. 5F). This distribution is complementary to that of the EphA3 receptor, which is concentrated in the temporal retina (Cheng *et al.*, 1995 and data not shown), but substantially overlapping with that of another receptor, EphA4 (Cek8), which is expressed in both the nasal (Fig. 5A) and temporal retina (Fig. 5B).

Polarized Tyrosine Phosphorylation of the EphA4 Receptor

Interestingly, although the expression levels of EphA4 that we detected in retinal ganglion cells were comparable in the nasal and temporal portions of the retina (arrows in Figs. 5A and 5B), as previously reported (Cheng *et al.*, 1995; Holash and Pasquale, 1995), in the temporal retina EphA4 transcripts were more concentrated in the retinal ganglion cell layer (Fig. 5B, arrow), whereas in the nasal retina they were evenly expressed in all the layers of the retina (Fig. 5A). The corresponding bright-field photographs (Figs. 5C and 5D) show that the Embryonic Day 8 nasal retina is less stratified, and therefore less differentiated, than the temporal retina, suggesting that EphA4 becomes preferentially expressed in the retinal ganglion cell layer as the retina differentiates. This is consistent with the decrease in overall EphA4 protein expression that we observed by immunoblotting Embryonic Day 6 to 10 retinal extracts (not shown).

Since ligand binding causes the Eph receptors to autophosphorylate on tyrosine residues (Davis *et al.*, 1994; Holash *et al.*, 1997; Pasquale, 1997) and activate cytoplasmic signaling pathways (Holland *et al.*, 1997), it is conceivable that Eph receptors that are expressed throughout the retina and optic tectum may exhibit polarized patterns of activation due to their partially overlapping distributions with activating ligands. Elucidating the patterns of Eph receptor activation, in addition to those of receptor expression, is crucial in order to understand the possible roles of Eph receptors and ephrins in the organization of the visual system. We have chosen EphA4 as a prototype to examine the tyrosine phosphorylation of Eph receptors in sectors of the retina with different ligand expression. The receptor EphA4 not only is expressed at similar and high levels in retinal ganglion cells from different parts of the retina (Cheng *et al.*, 1995; Holash and Pasquale, 1995), but also can bind ligands of the ephrin-A subclass, including ephrin-A5, as well as the ephrin-B subclass, including ephrin-B2 (Gale *et al.*, 1996).

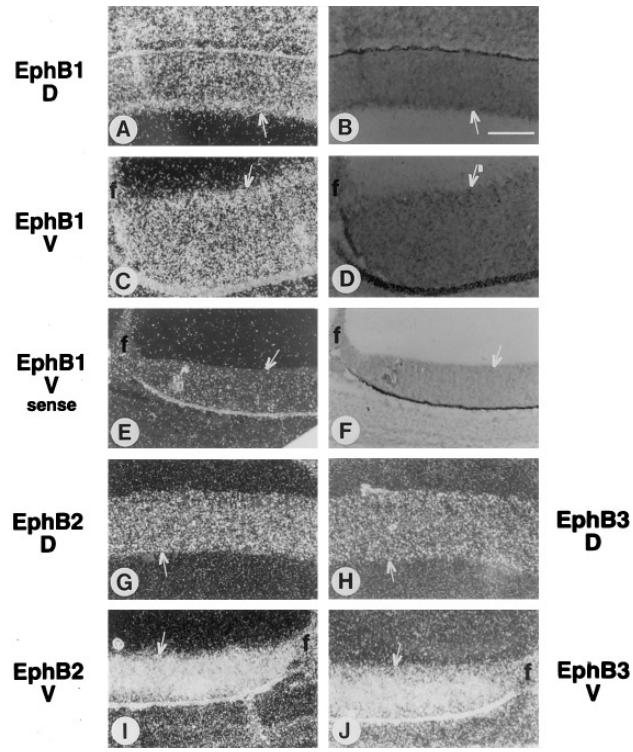
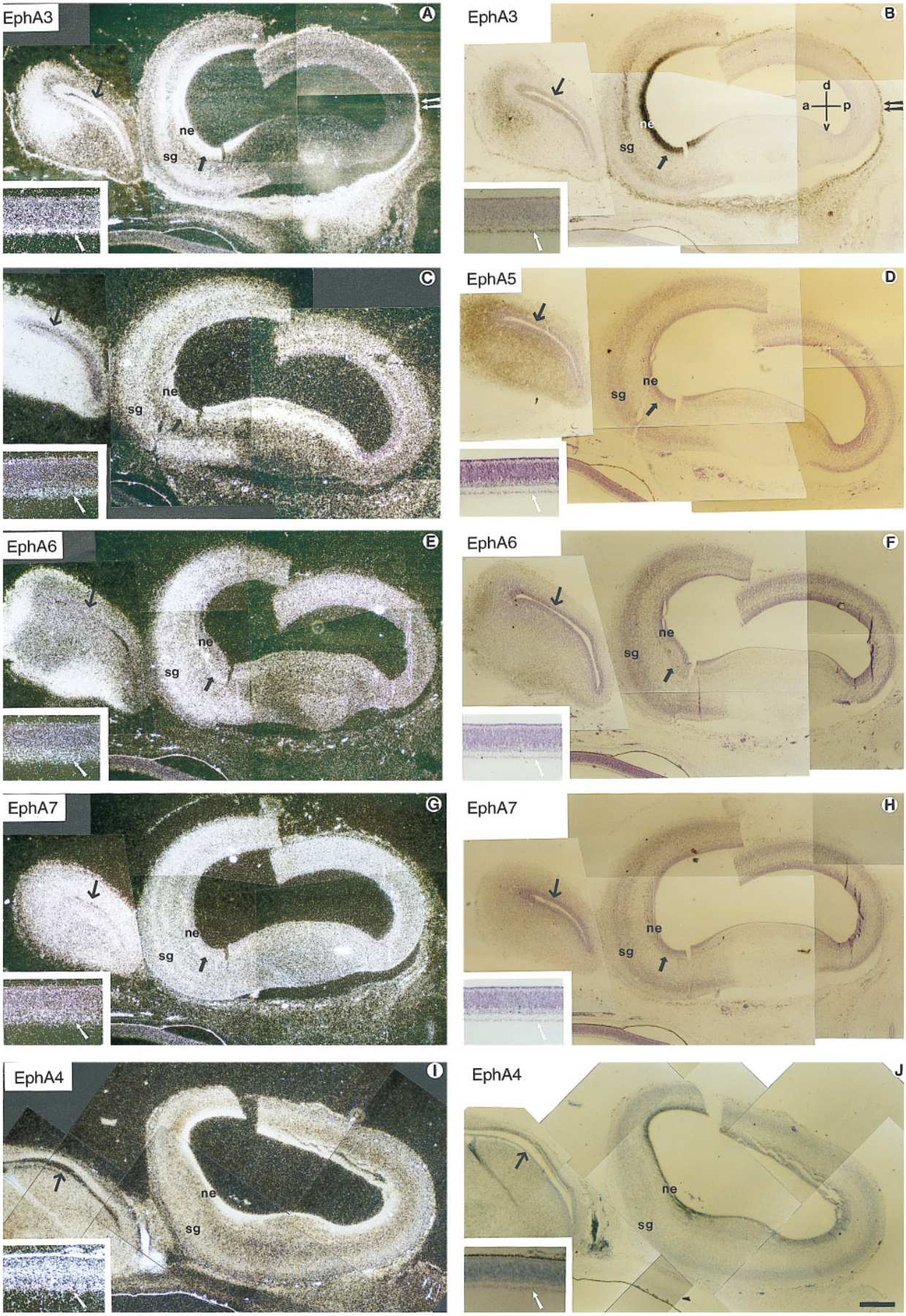


FIG. 3. Expression of EphB receptors in sagittal sections of the chicken retina at Embryonic Day 8. In dark-field photographs of *in situ* hybridization experiments, the antisense probe for EphB1 labeled the retinal ganglion cell layer (indicated by an arrow) in both dorsal (A) and ventral (C) retina. This labeling is specific, since it was not observed with the corresponding sense probe (E). The antisense probes for both EphB2 (G, I) and EphB3 (H, J) labeled the ventral retina (I, J) more prominently than the dorsal retina (G, H). The signals for EphB2 and EphB3 were distributed throughout the layers of the retina and not only in the retinal ganglion cell layer (indicated by an arrow). Bright-field photographs corresponding to A, C, and E are shown in the panels to the right (B, D, F). The position of the optic fissure is indicated (f) for orientation. The riboprobes used in these experiments are the same as those used in Fig. 2. D, dorsal; V, ventral. Scale bar in B, 150 μm.

EphA4 was immunoprecipitated with specific antibodies, and its level of phosphorylation on tyrosine was assessed by immunoblotting with anti-phosphotyrosine antibodies (Fig. 6). These experiments revealed that EphA4 is more phosphorylated in the dorsal than the ventral retina (Fig. 6A), consistent with activation by a ligand expressed dorsally, such as ephrin-B2. EphA4 was also more phosphorylated in the nasal than the temporal retina (Fig. 6C), consistent with a ligand expressed nasally, such as ephrin-A5. For comparison, we also probed EphA3 receptor immunoprecipitated from nasal and temporal retina with anti-phosphotyrosine antibodies (Fig. 6E). In contrast to EphA4, tyrosine phosphorylation of EphA3 was not detectable in either the nasal or the temporal retina (Fig. 6E), consistent with the low expression of ephrin-A5 in the temporal retina and the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low amount of receptor immunoprecipitated from the nasal retina (Fig. 6F).

Preliminary immunoprecipitation experiments showed that in Embryonic Day 8 optic tectum EphA4 is also phosphorylated on tyrosine, albeit at levels lower than in the retina, whereas tyrosine phosphorylation of EphA3 in the tectum is undetectable (not shown). These results are consistent with the observed overlapping distributions in the tectum of EphA4 and ephrin ligands and the complementary distributions of EphA3 and the ligands ephrin-A2 and ephrin-A5. The EphA3 and EphA4 receptors immunoprecipitated from the optic tectum are likely mostly of tectal origin, since substantial amounts of these receptors are present in the tectum not only at Embryonic Day 8 (Fig. 7), when some retinal axons are already present on the surface of the anterior tectum, but also at earlier stages of development (Fig. 7), when retinal axons have not yet begun to innervate the optic tectum (Goldberg, 1974).

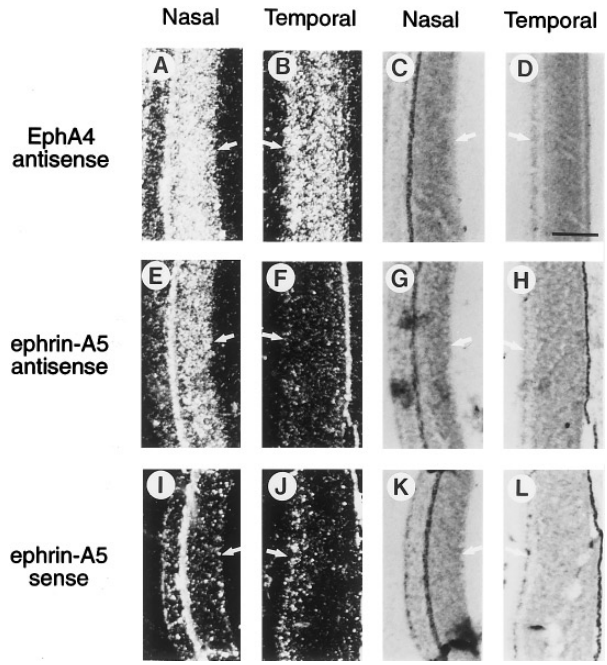

FIG. 5. Expression of EphA4 and ephrin-A5 in the Embryonic Day 8 retina. In dark-field photographs of *in situ* hybridization experiments, EphA4 was detected at approximately equal levels in the retinal ganglion cell layer (indicated by an arrow) of the temporal (A) and nasal (B) retina. EphA4 is preferentially expressed in the retinal ganglion cell layer in the more differentiated temporal retina (B) and is evenly distributed throughout the layers of the nasal retina (A). Ephrin-A5 is expressed throughout all layers of the nasal retina (E), but not the temporal retina (F). A control using the corresponding sense probe for Ephrin-A5 is also shown (I, J). For the identification of cell layers and determination of the level of differentiation, the corresponding bright-field photographs are also shown in the panels at right (C, D, G, H, K, L). Scale bar in D, 150 μm.

DISCUSSION

EphA3 and Ephrin-A Subclass Ligands

Although previous studies have focused on the expression of the receptor EphA3 in only retinal cells and the ligand ephrin-A5 in only tectal cells, we found that they are each expressed in both the retina and the tectum. Curiously, the high expression of EphA3 in temporal retina and anterior tectum and ephrin-A5 in nasal retina and posterior tectum (Fig. 8A) correspond to the patterns predicted by Sperry for chemoaffinity molecules mediating homophilic adhesion (Sperry, 1963). However, homophilic interactions appear to be an unlikely mechanism of guidance by EphA3 or ephrin-A5, since several Eph receptors and ephrins that have been examined do not mediate homophilic interactions (Böhme *et al.*, 1996; Holash *et al.*, 1997). Their distributions suggest that EphA3 and ephrin-A5 could regulate the organization of the visual system along the anterior–posterior axis in multiple ways. The graded expression of EphA3 in retinal cells and its ligands in tectal cells suggested the model previously proposed by others (Cheng *et al.*, 1995; Drescher *et al.*, 1996; Nakamoto *et al.*, 1996), which is based on a modification of the chemoaffinity theory that takes into account the ligand–receptor relationship of the molecules involved and the repulsive effects of the ligands toward receptor-bearing growth cones.

FIG. 4. Expression of EphA receptors in the optic tectum and temporal retina at Embryonic Day 8. In dark-field photographs of *in situ* hybridization experiments, EphA3 (A) is prominently concentrated in the neuroepithelial layer (ne) and developing stratum griseum centrale (sg) of the anterior tectum, the ventricular layer of the telencephalon (black downward arrow), a discrete area of the telencephalon, the meninges (double arrow), and the connective tissue underneath the tectum. In the bright-field photograph (B), where the signal (dark) is more discrete, EphA3 can also be detected in the thin subventricular layer (upward arrow) of the tectum. The EphA3 riboprobe used corresponds to nucleotides 748–1724; however, similar results were obtained with a probe corresponding to the region amplified with the degenerate primers. EphA5 (C) is expressed in the stratum griseum centrale (sg) and the subventricular layer of the tectum (upward arrow), and at high levels throughout the telencephalon with the exception of the ventricular layer. In at least the neuroepithelial layer, EphA5 appears to be in an increasing posterior to anterior gradient. EphA6 (E) was not detected at significant levels in the neuroepithelial layer of the tectum, while a discrete area of high expression is present in the telencephalon. EphA7 (G) was detected at low levels throughout the tectum and the telencephalon. In the temporal retina (insets) the receptors EphA3, EphA5, EphA6, and EphA7 were detected primarily in the retinal ganglion cell layer (white arrows). EphA4 (I) was detected most prominently in the neuroepithelial layer of the tectum and, at lower levels, in the stratum griseum centrale. At the right of each panel are the corresponding bright-field photographs (B, D, F, H, J) showing cresyl violet staining. Axes of the tectum are as indicated in B: a, anterior, p, posterior, d, dorsal, v, ventral. Scale bar in J, 500 μm.

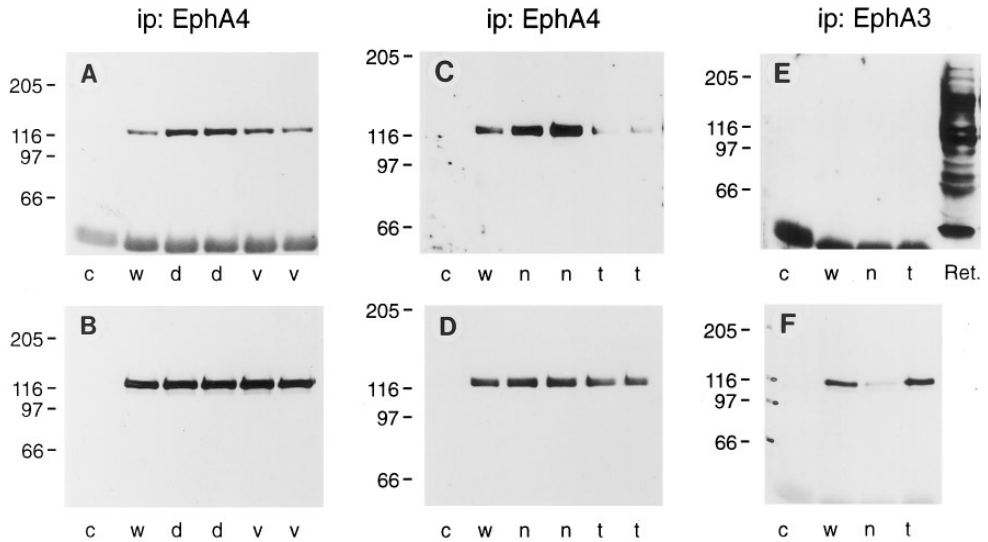

FIG. 6. Tyrosine phosphorylation of EphA4 in the Embryonic Day 8 retina. Immunoprecipitates (ip) of EphA4 (A, B, C, D) from whole (w), dorsal (d), ventral (v), nasal (n), or temporal (t) retina were probed using antiphosphotyrosine antibodies (A, C) and reprobed with anti-EphA4 antibodies (B, D). The immunoblots show that EphA4 in dorsal retina is more phosphorylated than in ventral retina (A), while EphA4 in nasal retina is more phosphorylated than in temporal retina (C). Approximately equal amounts of EphA4 were immunoprecipitated from dorsal and ventral retina (B), while a slightly greater amount of EphA4 was immunoprecipitated from nasal than temporal retina (D). EphA3 (E, F) was immunoprecipitated from whole (w), nasal (n), or temporal (t) retina, probed with anti-phosphotyrosine antibodies (E), and reprobed with anti-EphA3 antibodies (F). Substantial EphA3 was immunoprecipitated from temporal retina (F), but its level of phosphorylation on tyrosine was undetectable (E). A lane containing whole retina lysate (Ret.) is included in (E) as a positive control for the immunoblotting conditions used. The position of molecular mass standards, in kilodaltons, is indicated at the left of each panel.

In addition, however, the reciprocal expression patterns of EphA3 and its ligands in the retina and the tectum (Fig. 8A) could reflect roles in regulating compartmentalization of these structures along the anterior–posterior axis. This role would be similar to that proposed for the receptor EphA4 in defining rhombomere boundaries in the developing *Xenopus* hindbrain (Xu *et al.*, 1995). By controlling intraretinal and intratectal organization, EphA3 and its ligands could indirectly affect the organization of the retinotectal projection. For example, the reciprocal distributions of EphA3 and its ligands, ephrin-A2 and ephrin-A5, in both the neuroepithelial layer and stratum griseum centrale of the tectum (Monschau *et al.*, 1997) may underlie a role in restricting cell movement along the anterior–posterior axis of the tectum, and therefore contribute to the proper localization of ligand-expressing cells.

The reciprocal expression of EphA3 and ephrin-A5 in the retina may also underlie a more direct role in regulating the topography of retinal ganglion cell axons as well as efferent tectal projections, if both receptor and ligand are expressed on retinal axons. The localization of ephrin-A5 in axons would be consistent with the expected sorting of GPI-linked proteins to the axonal compartment of neurons (Rodriguez-Boulan and Powell, 1992). In zebrafish, a ligand closely related to ephrin-A5 has been reported to be expressed in nasal retinal ganglion cells and possibly in the optic nerve (Brennan *et al.*, 1997). Based on our localization of ephrin-A5 in the nasal retina, we propose that this ligand could represent the activity associated with nasal retinal ganglion cell axons that causes repulsion of temporal axons (Bonhoeffer and Huf, 1985; Raper and Grunewald, 1990). Interactions between retinal axons expressing EphA3 and retinal axons expressing ephrin-A5 could affect the organization of the axons while they extend on the inner surface of the retina, in the optic

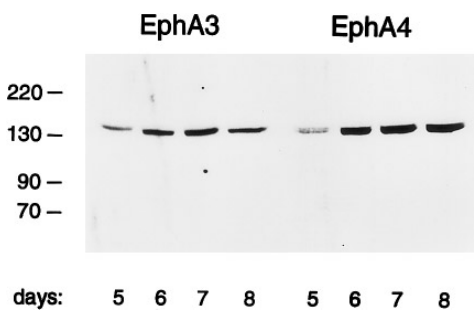

FIG. 7. Developmental regulation of EphA3 and EphA4 expression in the embryonic chicken optic tectum. Whole extracts of tectum at Embryonic Days 5, 6, 7, and 8 were probed with anti-EphA3 or anti-EphA4 antibodies. Equal amounts of protein were loaded in all the lanes. The position of molecular mass standards, in kilodaltons, is indicated at the left of each panel.

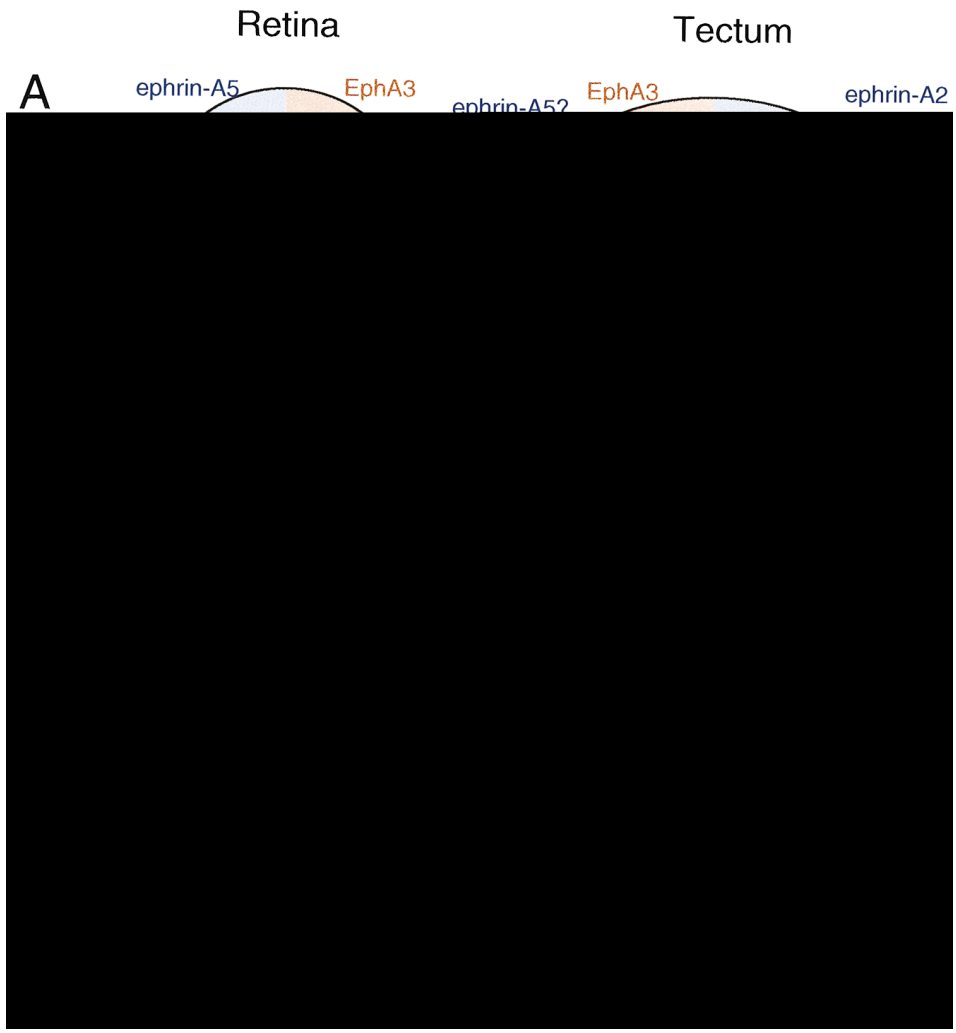

FIG. 8. Schematic representation summarizing the polarized patterns of expression or phosphorylation of some Eph receptors and ligands in the visual system. The known distributions of Eph receptors and ligands in the retina and the tectum are indicated by shading, question marks indicate hypothesized but not yet determined protein localizations in retinal axons. For simplicity, graded distributions are represented by even shading demarcating the hemisphere of higher expression. (A) Compartmentalization of the retina by the complementary distributions of the receptor EphA3 in the temporal retina (orange) and the ligand ephrin-A5 in the nasal retina (blue) and compartmentalization of the optic tectum by the complementary distributions of EphA3 in the anterior tectum and ephrin-A2 and ephrin-A5 in the posterior tectum. Temporal retinal axons, which express EphA3, do not grow into the posterior tectum, where ephrin-A2 and ephrin-A5 are localized. (B) Compartmentalization of the retina by the complementary distributions of the receptors EphB2 and EphB3 in the ventral retina (purple), and ephrin-B1 and ephrin-B2 in the dorsal retina (red). No polarized distributions of EphB receptors or transmembrane ligands have been identified so far in tectal cells. Receptors and ligands expressed on retinal ganglion cell axons could organize these axons in the optic nerve and/or along the dorsal–ventral axis of the optic tectum through fiber–fiber interactions. (C) The EphA4 receptor is uniformly expressed in retinal ganglion cells, but it overlaps with the ligand ephrin-B2 in the dorsal retina and the ligand ephrin-B5 in the nasal retina. The colocalization of EphA4 with its ligands could result in the pattern of activation indicated by blue shading: light blue indicating lower activation levels in the presence of only one ligand, dark blue indicating higher activation levels in the presence of two ligands simultaneously. This would result in an increasing ventral–temporal to dorsal–nasal gradient of EphA4 activation (phosphorylation), as indicated by the arrow.

nerve, and/or in the optic tract. Models have been proposed in which topography in the visual system is established through fiber–fiber interactions alone or in conjunction with other mechanisms (Willshaw and Von der Malsburg, 1979; Gierer, 1983; Fraser and Perkel, 1990). Although such models have received less attention than models based on interactions between retinal axons and the tectal surface, the existence of genes that regulate topography in the retina or the optic tract has been recently demonstrated in zebrafish (Kalstrom *et al.*, 1996; Trowe *et al.*, 1996).

EphA4 and Ephrin-A5

We have chosen EphA4 and ephrin-A5 as prototypes to investigate the mechanisms by which overlapping distributions of Eph receptors and ephrins may contribute to establishing axon topography. It is expected that colocalization of an Eph receptor and its ligand will cause tyrosine phosphorylation of the receptor and activation (Davis *et al.*, 1994; Holland *et al.*, 1997; Pasquale, 1997). Indeed, we found that the pattern of EphA4 phosphorylation on tyrosine correlates with the presence of ligands. Correlation between tyrosine phosphorylation of EphA4 and an increase in kinase activity has been previously demonstrated *in vitro* (Soans *et al.*, 1994). The determination of the sites of Eph receptor activation, rather than simply their sites of expression, is important to understand how individual Eph receptors are engaged in the organization of the retinotectal projection and possibly of tectal efferent projections.

The distributions of EphA4 and ephrin-A5 overlap in the nasal retina and the posterior tectum (Fig. 8C), suggesting that the receptor EphA4, although uniformly expressed along the axes of the retina and tectum, could influence the spatial organization of these structures by virtue of its polarized activation. Depending on the extent of overlap with its ligands, EphA4 could differentially regulate cell movement within the retina or the tectum, for example, by mediating changes in cytoskeletal organization or cell adhesion. Treatment of cultured retinal neurons with ephrin-A5 disrupted the organization of the actin cytoskeleton in growth cones (Meima *et al.*, 1997) and expression of the activated form of EphA4 disrupted cell adhesion in *Xenopus* embryos, possibly by affecting cadherin function (Winning *et al.*, 1996).

The pattern of EphA4 tyrosine phosphorylation in the Embryonic Day 8 retina is consistent with the superimposition of two ligand gradients that cause activation of EphA4 signaling pathways in the dorsal and anterior regions of the retina. Presumably, the ligand that activates EphA4 in the nasal retina is ephrin-A5, a high affinity ligand which we found to be expressed in the nasal but not the temporal retina. While these studies were in progress, ephrin-A5 was also reported to be present in the mouse and zebrafish nasal retina (Marcus *et al.*, 1996; Brennan *et al.*, 1997). Another ligand that may activate EphA4 is ephrin-B2, which was recently reported to be expressed in the dorsal mouse retina (Marcus *et al.*, 1996). *In vitro* binding assays have shown that ephrin-B2 exhibits appreciable affinity for EphA4 (Gale *et al.*, 1996). Thus, the phosphorylation pattern of EphA4 suggests that this receptor interacts *in vivo* with ligands of both the ephrin-A and ephrin-B subclasses, consistent with the *in vitro* studies (Gale *et al.*, 1996). As a result, the extent of EphA4 phosphorylation is expected to be different in each retinal quadrant: low in the ventral–temporal quadrant, intermediate in the ventral–nasal and dorsal–temporal quadrant, and high in the dorsal–nasal quadrant, where both ephrin-A5 and ephrin-B2 are present (Fig. 7C). EphA4 activation, consequently, increases along a ventral–temporal to dorsal–nasal axis.

EphA4 has been reported to be expressed on the axons of retinal ganglion cells throughout the optic pathway (Holash and Pasquale, 1995), where it could be differentially activated if its ligands are localized on subsets of axons (see above). Activation of EphA4 in retinal ganglion cell axons could differentially influence their growth rate and thereby the order in which they reach the tectum. *In vitro* experiments have suggested that ephrin-A5 selectively inhibits neurite outgrowth from caudally derived sensory and spinal neurons, which presumably express a receptor for this ligand (Donoghue *et al.*, 1996). Activation of EphA4 could also affect fasciculation and adhesion of retinal ganglion cell axons from different parts of the retina. Differences in fasciculation between temporal and nasal axons have been reported (Walter *et al.*, 1987). Recent evidence also suggests that ephrin-A5 promotes axon fasciculation in cultured cortical neurons (Winslow *et al.*, 1995). Interestingly, some of the mutations of zebrafish genes that affected the topography of retinal axons also influenced retinal axon fasciculation (Trowe *et al.*, 1996). Finally, the already enhanced activation of EphA4 in nasal retinal axons may reduce their responsiveness to low concentrations of ephrin-A subclass ligands present in the anterior and central tectum and thus allow these axons to proceed to their target regions in the posterior tectum without being repelled.

The colocalization of EphA4 and ephrin-A5 on nasal retinal axons, and the consequent activation of EphA4, predict that an additional novel mechanism may contribute to guiding these axons while they grow on the surface of the anterior tectum. Since the affinity of ephrin-A5 for EphA3 is approximately 10 times higher than that for EphA4 (Monschau *et al.*, 1997), it is conceivable that the activation of EphA4 in the nasal axons becomes downregulated as ephrin-A5 on the nasal axons preferentially interacts with EphA3 expressed at high levels by anterior tectal cells. Spatially regulated decreases of EphA4 activation would presumably have effects opposite to repulsion, thereby allowing, or even favoring, the growth of nasal retinal axons across the anterior tectum. In contrast, the high concentrations of ephrin-A5 in posterior tectal cells should increase EphA4 activation in nasal retinal axons and thereby inhibit their advancement, as previously proposed (Monschau *et al.*, 1997). We have detected tyrosine phosphorylation of EphA4 in the tectum (not shown), which may affect the organization of the tectum and its efferent projections. However, it will be important to determine whether the pool of EphA4 that is expressed in retinal ganglion cell axons extending on the surface of the tectum is activated. This will require immunohistochemical localization experiments with antibodies specific for a tyrosine phosphorylated epitope of EphA4.

Other EphA Receptors

EphA5, EphA6, and EphA7 transcripts are concentrated in retinal ganglion cells of the temporal retina and in layer II of the optic tectum. Because of the low expression levels of these receptors and the more advanced differentiation of the temporal retina and anterior tectum at Embryonic Day 8, it

was not possible to establish unequivocally whether these receptors are upregulated with differentiation or expressed in increasing anterior–posterior gradients. Expression of EphA5 has been previously reported to be even along the axes of the mouse retina (Zhang *et al.*, 1996; Meima *et al.*, 1997), while the putative zebrafish homolog of EphA7 is preferentially expressed in the temporal retina (Taneja *et al.*, 1996). Depending on whether they are expressed at significantly higher levels in the nasal compared to the temporal retina or in the posterior compared to the anterior tectum, these receptors could have distributions either complementary or overlapping with the distribution of ephrin-A5.

Since different subtypes of retinal ganglion cells are present in the retina (Mey and Thanos, 1992 and references therein), it is possible that different Eph receptors are expressed in different subsets of retinal ganglion cells. Although we did not attempt to compare the expression of different receptors at the single cell level, this possibility appears unlikely. Backlabeling of retinal ganglion cells by injecting rhodamine-labeled dextran into the rat superior colliculus indicated that all retinal ganglion cells, and not just a subset, express EphA5 (Meima *et al.*, 1997). Furthermore, in cultures of chicken retinal cells, most retinal ganglion cell neurites appear stained with anti-EphA3 antibodies, anti-EphA4 antibodies, or anti-EphA5 antibodies (Monschau *et al.*, 1997), suggesting that several Eph receptors are coexpressed in the same retinal ganglion cells. Retinal ganglion cell growth cones may thus be guided along the anterior–posterior axis of the optic tectum by the combined interactions of various EphA receptors with the ligands ephrin-A2 and ephrin-A5. Each receptor may transmit distinctive signals that are modulated according to its affinity for these ligands (Gale *et al.*, 1996; Monschau *et al.*, 1997).

EphB Receptors and Ephrin-B Subclass Ligands

Here we report that the receptor EphB3 is concentrated in neurons throughout the ventral retina with a pattern complementary to that of ephrin-B1 and ephrin-B2, which are concentrated in dorsal retinal neurons (Marcus *et al.*, 1996; Holash *et al.*, 1997) (Fig. 7B). Therefore, not only EphB2, as previously proposed (Holash and Pasquale, 1995; Kenny *et al.*, 1995), but also EphB3 may be involved in dorsal–ventral patterning of the retina. The reciprocal expression of EphB2 and EphB3 in the ventral retina and their ligands in the dorsal retina may restrict the movement of cells across the dorsal–ventral midline of the retina, a role similar to that proposed above for EphA3 and ephrin-A5 of restricting cell movement across the anterior–posterior midline of the retina and tectum.

EphB2 and EphB3 are more concentrated in the ventral than dorsal retina, but neither EphB receptors nor transmembrane ligands have been identified that are expressed in dorsal–ventral gradients in tectal cells (Holash *et al.*, 1997). This could reflect differences in the mechanisms by which Eph receptors guide retinal axons along the two axes of the tectum. EphB receptors expressed on the axons of ventral retinal ganglion cells, by interacting with transmembrane ligands expressed on the axons of dorsal retinal ganglion cells, could contribute to organizing retinal ganglion cell axons along the dorsal–ventral axis in the retina, optic nerve, and/or optic tract. Fiber–fiber interactions may play a more predominant role in maintaining the spatial organization of dorsal–ventral retinal ganglion cell axons, which arrive at the tectum roughly preordered with respect to their dorsal–ventral positions (Gierer, 1983; Thanos and Bohnoeffer, 1986) and grow along the anterior–posterior axis of the tectum while maintaining their dorsal–ventral relative positions (Baier *et al.*, 1996). Interestingly, stripe assays have not revealed guidance molecules differentially expressed in dorsal and ventral tectal cells (Walter *et al.*, 1987), while they have been instrumental for identifying repulsive molecules expressed in the posterior but not the anterior tectum (Stahl *et al.*, 1990; Drescher *et al.*, 1995). Molecules that provide dorsal–ventral directional information on the tectum appear to exist (Trowe *et al.*, 1996), but they could represent classes of molecules different from those providing anterior–posterior positional information on the tectal surface, which include ephrin-A subclass ligands and RGM, a 33-kDa GPI-linked glycoprotein that may act in close association with ephrin-A2 and ephrin-A5 (Müller *et al.*, 1996).

The distributions of the three EphB receptors examined hint to redundant functions for EphB2 and EphB3 and unique functions for EphB1. Interestingly, single gene mutations that affected the organization of ventral retinal axons have not been identified (Trowe *et al.*, 1996). A possible explanation for this is that more than one molecule functions to guide ventral retinal axons. Gene knock-out studies have also recently indicated that EphB2 and EphB3 may have redundant functions (Orioli *et al.*, 1996).

The distributions that we have found for Eph receptors in the developing retinotectal system are consistent with the model of guidance by contact-mediated repulsion that was proposed on the basis of the characterization of only one receptor, EphA3 (Cheng *et al.*, 1995; Drescher *et al.*, 1995). According to this model, Eph receptors located in the axons of retinal ganglion cells mediate repulsive responses to graded tectal guidance cues, which are represented by their ligands. Our results, however, suggest that the involvement of the Eph receptors in patterning of the visual system is considerably more complex, and may include guidance through fiber–fiber interaction mechanisms as well as indirect effects on retinotectal topography exerted by regulating the positional coordinates of cells within the retina and tectum. Furthermore, the distributions of Eph receptors and their ligands are not always complementary, but can overlap, resulting in persistent receptor activation and, therefore, possible novel mechanisms of axon guidance. This complexity may be required for precise regulation of axon guidance through different but cooperating mechanisms.

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